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PRINCIPAL INVESTIGATOR: Darryl Shibata, M.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, California 90033

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The earliest phases of breast cancer are poorly characterized. A precise understanding of the genetic changes which herald incipient transformation would be invaluable for accurate early diagnosis and a better understanding of its pathogenesis. Towards this goal, we aim to identify clonal breast populations based on the detection of only one X-chromosome androgen receptor allele after differential methylation sensitive restriction enzyme digestion. Although this analysis is suitable for large amounts of DNA, it was not reliable when small cell numbers were analyzed. Since the earliest breast cancer lesions are small, we have yet to develop a suitable assay for their analysis. However, new efforts based on technical improvements show promise for both high sensitivity and reliability.

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TITLE: GENETIC EVIDENCE OF EARLY BREAST CANCER

INTRODUCTION:

Cancer is thought to arise from a series of mutations which culminate in malignancy (1,2). The exact number, timing, and types of these mutations are unclear in breast cancer. Although the earliest genetic alterations in breast cancer are unknown, the earliest neoplastic lesions should be clonal. The goal of this proposal is to identify clonal regions of breast epithelium in order to further understand how early neoplasia arises. To accomplish this goal, we will analyze X-chromosome inactivation, since neoplastic regions should exhibit inactivation of the same X-chromosome whereas non-neoplastic regions should exhibit inactivation of both X-chromosomes.

Numerous studies have demonstrated that breast cancer is clonal based on X-chromosome inactivation studies (for example 3,4). This proposal seeks to extend these studies by analyzing both obvious tumor and the dysplastic, hyperplastic, and normal epithelium adjacent to the tumor. In this way, we seek to identify if the cancer arises from a generalized "field" of preneoplastic breast tissue. The normal and "premalignant" epithelium adjacent to primary tumors most likely includes some of the multi-step changes which precede trans-formation, and its analysis will help define the histologic characteristics and extent of this "field".

This topographic analysis requires the ability to microdissect at high resolution the thin layers of epithelium from surrounding stroma and tumor cells. The method for microdissection is selective ultraviolet radiation fractionation (SURF) (5). Small numbers (50-200) of cells (a single duct or lobule) with specific phenotypes (normal, premalignant, and tumor) (6) on a stained tissue section will be microscopically identified and covered with very small ink dots. UV radiation will then destroy everything except the DNA in the desired protected cells, and subsequent PCR should reveal their specific genotypes. X-chromosome inactivation provides the earliest evidence of clonal proliferation and can be used to identify clonal populations even if their underlying mutations are unknown (7,8). The topographical distributions of X-chromosome inactivation in the primary tumor, and its extension into adjacent non-neoplastic epithelium, can define the presence and extent of the altered epithelium which precedes transformation.

BODY:

Progress will be discussed in reference to the tasks identified in the proposal's statement of work:

STATEMENT OF WORK: GENETIC PROFILE OF EARLY BREAST CANCER

TASK 1: Optimize X-chromosome inactivation assay for SURF (Months 1-3)

A) Obtain female cell lines (N=4)
B) Make various mixtures of formalin fixed, paraffin embedded cell

lines
C) Compare results from SURF with DNA purified from cell lines

TASK 2: Determine the topographic distribution of X-chromosome inactivation in normal, premalignant, and malignant breast epithelium (Months 3-24)

A) Obtain fixed breast cancer specimens (40 per year)
B) Optimize X-chromosome analysis for PCR and SURF
C) Determine clonal patterns of X-chromosome inactivation in tumor tissue
D) Determine if the same clonal inactivation patterns extend into adjacent preneoplastic and normal epithelium
E) Analyze normal breast tissues

Task 1: We have obtained and isolated DNA from seven breast cancer cell lines (MCF-7, ER75, HEL1-8, BT474, MDAMB-453, MRF-7, MDA-BB) and have made artificial mixtures of known clonal compositions for analysis in Task 2.

Task 2 A: We have obtained formalin fixed tissue blocks from 40 breast cancer patients. They have been examined, and appropriate areas of tumor and adjacent normal tissue have been identified. The DNA has been extracted in bulk from the 40 breast cancers (both normal DNA and tumor DNA from the same patient).

Task 2 B: We have synthesized eight different PCR primers sets which span the methylation sensitive restriction enzyme sites (HpaII and HhaI) immediately 5′ to the triplets CAG repeats androgen receptor located on the X-chromosome. The primary method needed for this study is the ability to distinguish between polymorphic methylated androgen receptor loci. Restriction digestion using methylation sensitive enzymes (HpaII, HhaI) will cut only the unmethylated allele. Subsequent PCR with primers located outside of the restriction sites should only amplify the methylated (ie uncut) allele. Therefore, clonal populations are identified by the amplification of only a single allele whereas polyclonal (reactive) populations would yield both alleles (9-11).

Using various sets of these PCR primers, we have identified 10 out of the 40 breast cancers from Task 1 which are well suited for further analysis since they are polymorphic for the number of CAG repeats, with their two different alleles easily distinguished on small acrylamide minigels. We have been able to demonstrate clonal X-chromosome inactivation using restriction enzyme digestion and PCR in these breast cancers, using bulk extracted DNA.

A major problem has been encountered when the assay is scaled down to analyze the small amounts of DNA present in microdissected regions. With small numbers of cells (less than 1,000), the assay becomes unreliable with polyclonal populations demonstrating clonal patterns and clonal populations demonstrating polyclonal patterns. This lack of reliability with small numbers of cells is a major problem since the primary goal of this proposal is the detection of small clonal populations.

The primary problem with the current approach appears to be the inability of PCR to accurately represent the proportion of alleles present in the original sample. Because small numbers of cells must be analyzed, a large number of PCR cycles (40-46) are necessary to achieve the necessary sensitivity. Unfortunately, if a minor residual fraction of one androgen receptor allele is present (still indicating a clonal population) the large number of PCR cycles obscures the true starting fraction, and both alleles are amplified to similar extents, leading to "false" negatives.

The contamination of clonal populations by the "unmethylated" allele is due to two sources. First, all breast cancers are contaminated by normal stroma cells. Even with microdissection, approximately 5-10% contamination by stroma cells is inevitable. Second, restriction digestion may be incomplete leading to a small fraction of uncut but unmethylated alleles. Therefore, clonal breast epithelial populations will always harbor a small fraction of contaminating "nonclonal" alleles.

Another problem is with polyclonal populations. Because formalin fixed, paraffin embedded tissues are used, DNA degradation is present. The extent of this degradation cannot be predicted and sometimes only a small number of molecules can be amplified. However, if only a small number of DNA molecules are suitable for PCR amplification, then the same allele may be selected by chance from even polyclonal populations. Therefore, using known reactive and monoclonal (ie tumor) tissues, reliable detection has not been possible.

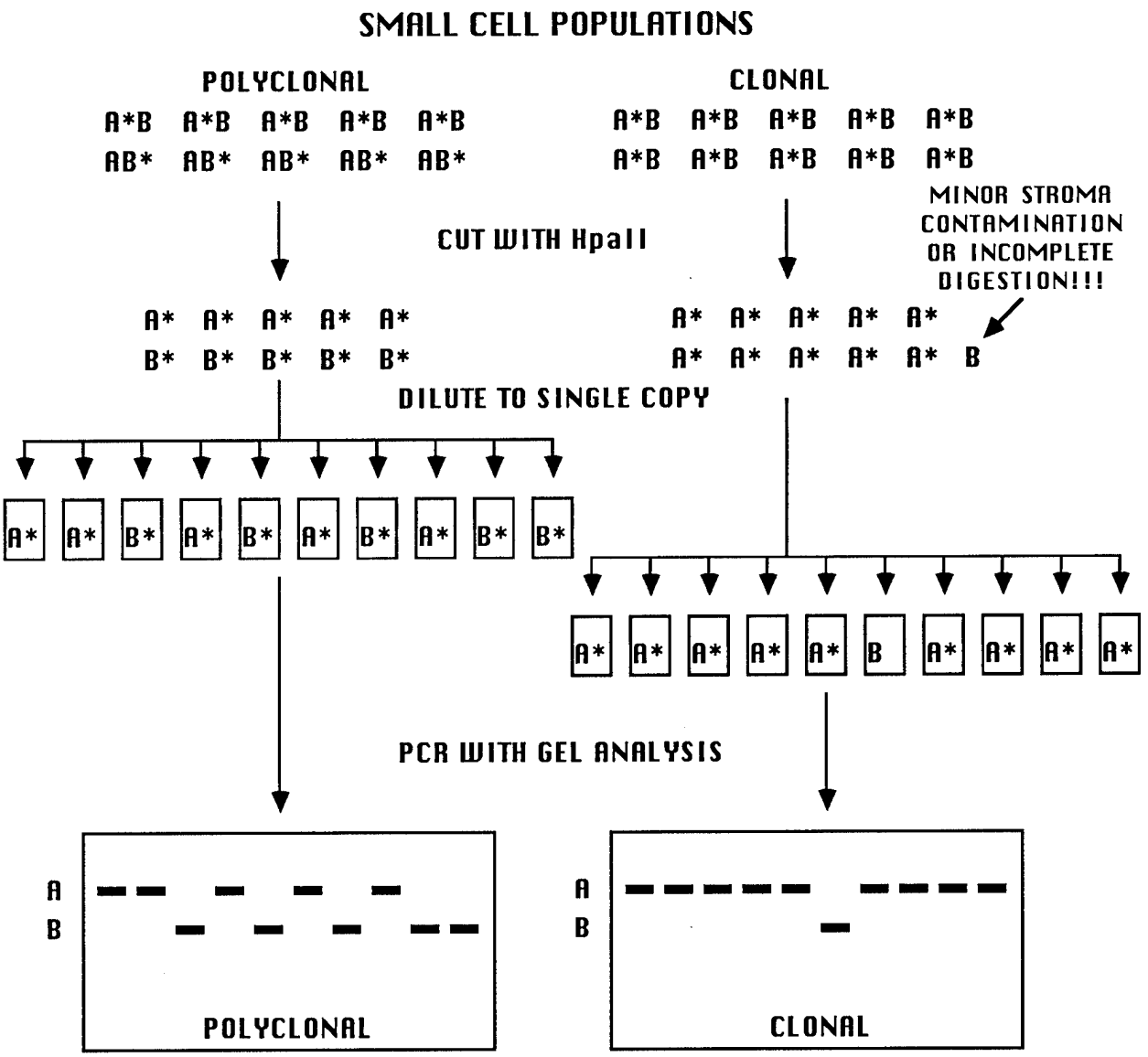
To overcome these informative failures, we have changed the strategy. Although PCR can amplify small numbers of alleles to detectable levels, the primary problem is the inability of PCR to accurately represent the proportion of alleles present in the original sample. Therefore, we have altered the assay such that PCR is no longer used to distinguish between different allelic proportions. Instead, separation of the alleles occurs prior to PCR and PCR is only used to detect the alleles. This is illustrated below:

STRATEGY

A) Isolate DNA from a small number of cells
B) Cut with HpaII or HhaI
C) Dilute to single copy (approximately 1 copy per PCR tube)
D) PCR to detect single copy
E) Gel analysis to identify allele based on size
F) Count numbers of each allele
CLONAL= >70% or <30% of each allele
POLYCLONAL= Each allele between 30-70%

ADVANTAGES:

1) Ability to "count" alleles avoids "false" positives due to analysis of too few alleles
2) Allows statistical analysis to identify clonal populations
3) Easily interpreted compared to bulk DNA analysis which requires judgment on whether a given PCR band is stronger than another.


SMALL CELL POPULATIONS
POLYCLONAL
A*B A*B A*B A*B A*B
AB* AB* AB* AB* AB*
CLONAL
A*B A*B A*B A*B A*B
A*B A*B A*B A*B A*B
MINOR STROMA CONTAMINATION OR INCOMPLETE DIGESTION!!!
CUT WITH HpaII
A* A* A* A* A*
B* B* B* B* B*
A* A* A* A* A*
A* A* A* A* A* B
DILUTE TO SINGLE COPY
A* A* B* A* B* A* B* A* B* B*
A* A* A* A* A* B A* A* A* A*
PCR WITH GEL ANALYSIS
A
B
POLYCLONAL
A
B
CLONAL

Task 2, C,D,E: These tasks have not been accomplished as they are dependent on the success of Task 2B, as noted above.

CONCLUSIONS:

Although problems have been encountered, a new approach should provide the highly sensitive and reliable assay necessary to detect clonal breast epithelium. We feel the proposed assay is innovative and improves on prior methods to detect small clonal populations. Once this assay is optimized, investigations should proceed rapidly to identify early clonal breast epithelial populations.

REFERENCES:

1.Nowell PC: The clonal evolution of tumor cell populations. Science 194:23-28, 1976
2.Bishop JM: The molecular genetics of cancer. Science 245:305-11, 1991
3.Noguchi S, Motomura K, Inaji H, Imaoka S, Koyama H. Clonal analysis of fibroadenoma and phyllodes tumor of the breast. Cancer Res 1993;53:4071-74
4.Noguchi S, Motomura K, Inaji H, Imaoka S, Koyama H. Clonal analysis of human breast cancer by means of the polymerase chain reaction. Cancer Res 1992;52:6594-97
5.Shibata D, Hawes D, Li ZH, Hernandez AM, Spruck CH, Nichols PW: Specific genetic analysis of microscopic tissue after selective ultraviolet radiation fractionation and the polymerase chain reaction. Amer J Pathol 141:539-43, 1992
6.Page DL, Simpson JF. Benign, high-risk, and premalignant lesions of the mamma. In, Bland KI, Copeland EM (eds) The Breast, WB Saunders Co, Philadelphia 1991, pp113-134
7.Fearon ER, Hamilton SR, Vogelstein B. Clonal analysis of human colorectal tumors. Science 1987;238:193-7.
8.Lyon MF. X-chromosome inactivation and developmental patterns in mammals. Biol Rev 1972;47:1-35
9.Tilly WD, Marcelli M, Wilson JD, McPhaul M. Characterization and expression of a cDNA encoding the human androgen receptor. PNAS 1989;86:327-31
10.Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X-chromosome inactivation. Am J Hum Genet 1992;51:1229-39
11.Mashal RD, Lester SC, Sklar J. Clonal analysis by study of X chromosome inactivation in formalin-fixed paraffin-embedded tissue. Cancer Res 1993;53:4676-9